Non-targeted metabolite profiling highlights the potential of strawberry leaves as a resource for specific bioactive compounds

Kårlund, Anna; Hanhineva, Kati; Lehtonen, Marko; McDougall, Gordon J.; Stewart, Derek; Karjalainen, Reijo O.

Published in:
Journal of the Science of Food and Agriculture

DOI:
10.1002/jsfa.8027

Publication date:
2017

Document Version
Peer reviewed version

Non-targeted Metabolite Profiling Highlights the Potential of Strawberry Leaves as a Resource for Specific Bioactive Compounds

Anna Kårlund[*,†], Kati Hanhineva[†], Marko Lehtonen[‡], Gordon J. McDougall[#], Derek Stewart[#,ø] and Reijo O. Karjalainen[§,†]

[†] Institute of Public Health and Clinical Nutrition, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland

[‡] School of Pharmacy, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland

[#] The James Hutton Institute, Invergowrie, Dundee DD2 5DA, Scotland UK

[ø] Norwegian Institute of Bioeconomy Research, Nord Holt, Postboks 2284, 9269, Tromsø, Norway

[§] Department of Environmental and Biological Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland

*Correspondence: Tel.: +358 50 371 1421; Fax +358 17 162 131

E-mail: anna.karlund@uef.fi

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.8027

ABSTRACT

BACKGROUND: The non-edible parts of horticultural crops, such as leaves, contain substantial amounts of valuable bioactive compounds which are currently only little exploited. For example strawberry (*Fragaria* × *ananassa*) leaves may be a promising bioresource for diverse health-related applications. However, product standardization sets a real challenge, especially when the leaf material comes from varying cultivars. The first step towards better quality control of berry fruit leaf-based ingredients and supplements is to understand metabolites present and their stability in different plant cultivars, so we surveyed the distribution of potentially bioactive strawberry leaf metabolites in six different strawberry cultivars. Non-targeted metabolite profiling analysis using LC-qTOF-ESI-MS with data processing via principal component analysis and *k*-means clustering analysis were utilized to examine the differences and commonalities between the leaf metabolite profiles.

RESULTS: Quercetin and kaempferol derivatives were the dominant flavonol groups in strawberry leaves. Previously described and novel caffeic and chlorogenic acid derivatives were among the major phenolic acids. In addition, ellagitannins were one of the distinguishing compound classes in strawberry leaves. In general, strawberry leaves also contained high levels of octadecatrienoic acid derivatives, precursors of valuable odor compounds.

CONCLUSIONS: The specific bioactive compounds found in the leaves of different strawberry cultivars offer the potential for the selection of optimized leaf materials for added-value food and for non-food applications.

KEYWORDS: *Fragaria* × *ananassa*, leaves, non-targeted metabolite profiling, polyphenols, cultivars

INTRODUCTION

Agricultural and food industry byproducts constitute an abundant resource of bioactive and functional ingredients including natural antioxidants and antimicrobial compounds that are potentially applicable in the products of food, pharmaceutical and cosmetic industries.[1-3] Leaves are one of the largest side streams of berry fruit production, and their enhanced utilization level would be beneficial in improving the sustainability of agricultural practices.[3] Furthermore, intelligent utilization of this "waste material" may bring added value at both ends of the production chain: from primary production of crops to the functional food or non-food applications. It has been reported that leaves of berries and fruits are rich in putatively bioactive polyphenols: high concentrations of polyphenols have been measured from leaves of e.g. strawberry (*Fragaria* × *ananassa*)[4,5], currants (*Ribes* spp.)[3,6], raspberry (*Rubus ideaus*), honeysuckle (*Lonicera kamtschatica*)[7], lingonberry (*Vaccinium vitis-idaea*)[8], apple (*Malus domestica*), chokeberry (*Aronia melanocarpa*), cranberry (*Vaccinium macrocarpon*), bilberry (*Vaccinium myrtillus*)[2], and saskatoon (*Amelanchier alnifolia*)[9].

Strawberries are widely cultivated horticultural crops possessing a large array of compounds with putative bioactivities and functionalities; the major compound groups in strawberry fruits include flavonoids, hydrolyzable and condensed tannins, organic and phenolic acids, fatty acids, and terpenoids.[10,11] Emerging evidence from human trials suggest[12] that intake of these compounds as part of fruit may enhance body defences against oxidative damage[13], may suppress baseline formation of oxidants by circulating phagocytes[14], and may beneficially influence the blood lipid profile by reducing total cholesterol, low-density lipoprotein cholesterol and triglycerides levels[15]. Thereby, the intake of bioactive compounds of strawberry fruit may help to lower the overall risk of cardiovascular diseases.

Besides the fruit, strawberry leaves may also be a promising bioresource for diverse health-related applications, as they are rich in a wide range of potentially bioactive compounds, and the antioxidant capacity of strawberry leaves has been found to be considerably higher than in berries.[16] Furthermore, the extract of wild strawberry leaves has been reported to exhibit endothelium-dependent vasodilator activity[17], protective activity against diabetic nephropathy[18], and modulation of inflammation and autophagy related markers[19] in model systems. Phytochemical-rich extracts also possess strong antimicrobial activity, and strawberry leaf extracts may therefore be a suitable feedstock for the development of food preservatives.[5]

Cultivation, storage and processing conditions, harvesting season, as well as the genetic background of the plant source, affect the chemical composition of byproduct-based raw materials.[1,3] Hence, product standardization sets a real challenge, especially when the plant material represents varying cultivars and is collected from several locations.[1] The first step towards better quality control of berry fruit leaf-based ingredients and supplements is to survey the metabolite profiles and their stability in different berry fruit cultivars. Furthermore, knowledge gained regarding the bioactive/functional compounds from the analysis of leaf materials will ease the branding of specific end-products. [20]

Non-targeted, liquid chromatography and mass spectrometry-based metabolite profiling offers a hypothesis-free approach for the analysis of plant metabolites beyond traditional, targeted profiling of polyphenolic compounds. In this study, analysis with liquid chromatography connected with negative electrospray ionization in quadrupole–time-of-flight–mass spectrometry (LC-qTOF-ESI-MS) was utilized to separate and identify major compounds in the leaves of six different European strawberry cultivars grown in commercial farms in Eastern Finland. Data processing by principal component

analysis (PCA) and *k*-means clustering (KMC) analysis of the levels of the main metabolite groups unveiled both differences and commonalities between the leaf metabolite profiles of strawberry cultivars.

EXPERIMENTAL

Leaf samples

Leaf samples (40-80 g) from 6 strawberry (*Fragaria* × *ananassa*) cultivars (Florence, Honeoye, Jonsok, Polka, Rumba, and Salsa) were collected from three commercial strawberry farms in Northern Savonia, Eastern Finland on June 5th 2013, before full fruiting season. Two farms were located in Leppävirta (62.30°N, 27.475°E). Farm 1 produced Florence, Jonsok, Polka, and Salsa, and Farm 2 produced Honeoye. The third farm was located in Karttula (62.53°N, 26.58°E) and produced Rumba. For three days before harvesting, the weather was dry in all locations, and the average temperature was 21.6 °C (13.2-29.1 °C) and 20.0 °C (10.7-27.5 °C) in Leppävirta and Karttula, respectively. Sand moraine is the predominant soil type in Northern Savonia. Farmers did not report any significant pest damage during the season.

For each cultivar, three biological sample replicates were collected with each replicate gathered from 10-20 individual strawberry plants. Fully developed leaves were cut with scissors. After collection, samples were immediately placed on ice and frozen as soon as possible. Leaves were stored at -20 °C until further processing.

Non-targeted metabolite profiling

Sample preparation. For the metabolite extraction, 10 frozen leaves (14-37 g) for each cultivar were ground in mortar with liquid nitrogen; three biological sample replicates (i.e. 3 × 10 leaves) were prepared for each cultivar. Compounds were

extracted with 800 mL L^{-1} methanol; 30 mL for 10 g of leaf powder.[21] Suspensions were vortexed at high speed and then extracted in a horizontal shaker (Unimax 2010, Heidolph Instruments, Schwabach, Germany) at 400 rpm for 15 min at room temperature. Extracts were filtered through gauze and centrifuged (Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 3220 g for 5 min. The clarified supernatants were stored at -75 °C until analysis. Prior to the metabolite analysis, samples were again centrifuged and filtered (Acrodisc® 0.22 μm PTFE filter, PALL, Port Washington, NY, USA).

The total phenolic contents of strawberry extracts were measured by the Folin-Ciocalteu method[22], with some modifications. Briefly, 200 μL of diluted leaf extract (1:50 or 1:100) was combined with 1000 μL of 100 mL L^{-1} (v/v) Folin-Ciocalteu reagent (VWR International, Darmstadt, Germany) and 800 μL of 75 g L^{-1} (w/v) Na_2CO_3 (Sigma-Aldrich, St. Louis, MO) solution; ultrapure water was used as the blank sample. The reagent mixture was incubated at room temperature for 60 min and vortexed every 20 min. The mixture absorbance was measured at 765 nm (Ultrospec 2000, Pharmacia Biotech, Uppsala, Sweden) and the total phenolic content was calculated as gallic acid (GA) (Sigma-Aldrich, St. Louis, MO) equivalent according to standard curve (0.01, 0.03, 0.05, 0.07, and 0.1 mg GA /mL).

LC-MS conditions. The LC-MS conditions have been described in detail previously.[11] Briefly, the samples were analyzed by LC-qTOF-ESI-MS (Agilent Technologies, Waldbronn, Karlsruhe, Germany) consisting of a 1290 LC system, a Jetstream electrospray ionization (ESI) source, and a 6540 UHD accurate-mass qTOF spectrometer. The samples were spearated by reversed phase (RP) chromatography and data were acquired in negative electrospray ionization (ESI–).

Two microliters of the sample solution were injected onto a column (Zorbax Eclipse XDB-C18, 2.1 × 100mm, 1.8 μm) (Agilent Technologies, Palo Alto, CA, USA) kept at 50 °C. Mobile phases, delivered at 0.4 ml/min, consisted of water (eluent A) and methanol (eluent B) (Sigma-Aldrich, St. Louis, MO), both containing 1 mL L^-1 (v/v) of formic acid (Sigma-Aldrich, St. Louis, MO). For ESI data acquisition, 2 GHz extended dynamic range mode was used and the instrument was set to acquire over the m/z 20–1600. For automatic data dependent MS/MS analyses, precursor isolation width was 1.3 Da, and from every precursor scan cycle, 4 most abundant ions were selected for fragmentation. Collision energies were 10, 20 and 40 V in subsequent runs. The sample tray was kept at 4 °C during the analysis.

Data processing. MassHunter Qualitative Analysis B.05.00 (Agilent Technologies, Palo Alto, CA, USA) software was used for the collection of the data matrix. Ions were combined to molecular features exhibiting isotopic peaks, dimers, and common adducts. The collected data was exported to Mass Profiler Professional software (version 2.2, Agilent Technologies, Palo Alto, CA, USA) for compound alignment across all measured samples. Noise and low abundance metabolites were removed from the data matrix according to their abundance and appearance frequency in the samples: only compounds present in all three sample replicates of at least one sample type were examined, and the final dataset contained 1168 molecular features.

For the comparison of the abundances of molecular features, the data matrix was exported to Excel and sorted based on the maximum peak area abundance values. Molecular features having a maximum peak area over 500 000 were selected for further investigations, which reduced the dataset to comprise 387 molecular features. We have previously conducted metabolite profiling from strawberry flowers[23] and strawberry fruits[11] and these studies provided a database for the annotation of abundant strawberry

leaf compounds. Previously reported fragmentation patterns in the data-dependent MS/MS acquisition were used to putatively identify potential compounds. Some compounds were annotated on the basis of their molecular weight and retention time. In addition, several molecular features were compared against The METLIN Metabolite Database (http://metlin.scripps.edu/), SciFinder (https://scifinder.cas.org), or other earlier published work describing fragmentation patterns[24,25]. Consequently, 84 metabolites were tentatively annotated.

Statistical analyses. Principal component analysis (PCA) (Simca 13.0, Umeå, Sweden) was utilized in order to demonstrate the relationship between samples and sample replicates in their metabolite profiles using the full data set of 1168 molecular features. *k*-Means clustering (KMC) analysis with heat map representation (TM4 Microarray Software Suite, available at. www.tm4.org/mev.html; algorithm according to Soukas et al.[26]) was applied to analyze and display the accumulation patterns of the 84 annotated major compounds, and to visualize the differences in normalized metabolite signal abundances between samples types. Compounds were grouped to 10 clusters. Differences among the total phenolic contents of strawberry leaf samples (three biological replicates per cultivar; three technical replicates per biological sample) were analyzed by analysis of variance together with Tukey's t-test and Tamhane test ($p<0.05$) (SPSS 16.0, SPSS Inc., H, Chicago, IL).

RESULTS AND DISCUSSION

The major strawberry leaf metabolites show both universal and cultivar-dependent distribution and reflect genetic factors

We tentatively identified 84 major potential metabolites from the leaves of six strawberry cultivars (Table 1). According to LC-MS abundance, the main compound

groups present in strawberry leaves included flavonols, phenolic acids, and fatty acids and their derivatives, along with flavan-3-ol derivatives, ellagitannins (i.e. galloyl hexose derivatives) and terpenoids. In addition, other miscellaneous metabolites, such as aromatic and aliphatic carboxylic and dicarboxylic acids, were annotated. Quercetin and kaempferol derivatives were the dominant flavonols, whilst caffeic, chlorogenic, and coumaric acid derivatives were the major phenolic acids.

We previously reported most of the phenolic and terpenoid compounds annotated here in the flowers[23] and fruits[11,27] of strawberries. For example flavonoids, ellagitannins, terpenoids and flavan-3-ol derivatives are important factors in the reproduction, development, and defence mechanisms of strawberries, and hence, they can be expected to be found also in strawberry leaf tissues. These compound classes are important for the functional and sensory properties of strawberry-based products.

The results of the present study are also well in line with previous studies investigating the phenolic composition of berry fruit leaves, as flavonols, hydrolyzable and condensed tannins and hydroxycinnamic acids (such as chlorogenic acids) have emerged in the leaves of black currant (*Ribes nigrum*)[6], honeysuckle, raspberry[7], bilberry, lingonberry[8], and saskatoon[9]. Overall, the main flavonols in many berry fruit leaves were predominantly quercetin and kaempferol derivatives.[7] Oszmiański et al.[7] analyzed the phenolic composition of strawberry leaves and found that the main compounds were coumaroyl glucoside, ellagic acid, quercetin-3-*O*-glucuronide-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucuronide.

In the principal component analysis (PCA) (Fig.1.), the first principal component and the second principal component explained 23.0 % and 16.7 % of the variation in the metabolite profiles, respectively. The samples were clustered mainly according to their

replicates, which indicates high intra-cultivar stability of the chemical composition of strawberry leaves.

The results of PCA also indicate that there is inter-cultivar and growing season-related variation in the metabolic profiles of strawberry leaves. The June bearing cultivars Honeoye and Rumba were closely situated in the PCA plot. Honeoye and Rumba seem to serve the same role as early strawberry season varieties[28], and the physiological properties that are essential for the plant fitness in the early season may explain the similarities in the metabolite profiles of these cultivars. All other cultivars were markedly separated from each other in the PCA.

Quercetin and kaempferol derivatives exhibited variable levels in strawberry leaves

Several quercetin derivatives were identified as major strawberry leaf metabolites (Table 1). Overall, quercetin derivatives showed variable levels in the leaf samples (Fig.2., Fig.3.). Likewise, kaempferol derivatives demonstrated variability and cultivar-dependent patterns of appearance.

Quercetin is the most common flavonol present in edible plants, and quercetin glucuronides are typical compounds in the leaves of berries and fruits. Importantly, in line with the present study, Oszmianski et al.[7] previously found that quercetin derivatives are the most abundant class of low molecular weight phenolic compounds in strawberry leaves.

Quercetin glucuronides are also formed by human metabolism after administration of quercetin from food: quercetin-3-*O*-β-glucuronide is the main quercetin metabolite in the blood stream, and it has many physiological functions.[29] Due to its phenolic hydroxyl groups, quercetin displays relatively high antioxidative potential, and

quercetin-3-*O*-β-glucuronide is suggested to be concentrated to a target tissue under oxidative stress.[29] Actually, quercetin-3-*O*-β-glucuronide may act as a detoxified form of quercetin aglycone, the compound that finally exerts the bioactivity on the target site.[29] The matrix and dose with which quercetin glucuronides are introduced to the body impact on quercetin accumulation in tissues.[29] Most importantly, the conjugation position affects the biological activity of quercetin glucuronides.[30]

Like quercetin, kaempferol derivatives are widely distributed in plant kingdom and hence, they are usually abundant in human diet.[31] Epidemiological studies have implied that high intake of kaempferol-rich foods, such as strawberries, reduces the incidences of some types of cancer and cardiovascular diseases.[31] Kaempferol is extensively metabolized to kaempferol glucuronides after oral administration, and shows moderate or low absorbtion.[32] However, dietary kaempferol derivatives have been suggested to be absorbed more efficiently than quercetin derivatives in humans, at least from specific food matrices.[33] Due to the overall high quercetin and kaempferol derivative content, strawberry leaves would appear to be a promising feedstock for bioactive/functional, flavonol-rich ingredients and supplements.

Ellagitannins are an abundant, potentially bioactive class in strawberry leaves

The leaves of cultivars Florence and Salsa demonstrated especially high levels of different classes of hydrolyzable tannins (Fig.2., clusters 1 and 7, respectively). Although negative ion fragmentation data was not available for some potential ellagitannins and the upper limit of *m/z* acquirement was set to 1600 which misses some high molecular weight ellagitannins, ellagitannins were clearly one of the distinguishing compound classes in strawberry leaves.

Indeed, in a targeted, quantitative analysis of the phenolic composition of the leaves of greenhouse-grown Polka strawberries, ellagitannins were the most abundant compound group; flavonoids and proanthocyanidins were the second and third abundant compounds, respectively.[5] Ellagitannins occur in high levels also in strawberry fruits.[10] Sanguiin H-6, lambertianin C, galloyl bis-hexahydroxydiphenic acid (HHDP) glucose[10], agrimoniin, and five other ellagitannins[34] have been detected in fruits of different strawberry cultivars.

A large body of experimental data on the potential health benefits of ellagitannins is based mainly on *in vitro* and animal models. However, it is well demonstrated that the bioavailability of ellagitannins or ellagic acid is very low, but that urolithins, the metabolites of ellagitannins produced by gut microbiota, are much better absorbed. Limited human trials suggest that urolithins may exert antioxidant, anti-inflammatory, anticarcinogenic and antimicrobial activities (reviewed by Espín et al.[35]).

Potentially bioactive caffeic acid derivatives were identified from strawberry leaves

Previously annotated and novel caffeic and chlorogenic acid derivatives were among the major phenolic acids identified in strawberry leaves (Table 1). For example caffeoyl threonate and caffeoyl shikimate were tentatively annotated, and were observed to be in relatively high levels in cultivars Polka (cluster 10) and Jonsok (cluster 4), respectively (Fig.2.). Caffeoyl threonates have been previously identified from the leaves of *Crataegus* spp. (Rosaceae) and aroused some interest due to their possible role in the therapeutic effects of *Crataegus* preparations in cardiac diseases.[36] Caffeoylshikimic acid has been previously found in the leaves of lingonberry, bilberry, and hybrid bilberry (*Vaccinium* × *intermedium* Ruthe)[8], and it is proposed to be an

intermediate in the pathway for chlorogenic acid synthesis[37]. In addition to caffeoyl shikimate, some chlorogenic acid derivatives were on high levels in Jonsok (Fig.2., cluster 4). Chlorogenic acid is a strong antioxidant[37] and an antimicrobial agent[38], and its concentration in strawberry leaves may be further increased with specific elicitors[5].

Octadecatrienoic acids, precursors of valuable odor compounds, are abundant in strawberry leaves

Several fatty acids, especially octadecatrienoic acid derivatives (Table 1), were generally abundant in strawberry leaves (Fig.2.). Relatively high and consistent levels of linoleic and linolenic acids (cluster 3) were detected in Honeoye whilst Jonsok had the highest level of oleic acid (cluster 9). Octadecatrienoic acid derivatives other than linolenic acid showed variable levels of abundance.

Octadecatrienoic acids are 18-carbon (C18) polyunsaturated fatty acids, and can be enzymatically converted to other octadecatrienoic acids.[39] Furthermore, C18-fatty acids function as precursors for aromatic green leaf volatiles, C6 and C9-metabolites that are associated with the green notes of fruit and vegetable odor.[39] C6-volatiles are widely used in food and beverage industry, and they represent one of the most valuable flavor classes.[39] C6 and C9-green leaf volatiles also have some antimicrobial potential that could be utilized in food preservation applications.[39,40] Although the C6 and C9-metabolites of C18 fatty acids can be synthesized chemically, consumers are increasingly showing a preference for more natural additives, e.g. in aromas and preservatives.[39,40] In addition, strawberry leaves have an active enzyme system that produces high quantity of C6-aldehydes and this system can be utilized to produce green leaf essence from linolenic acid.[41] This fits with the increasingly important Green Chemistry agenda and the protection of human health as outlined in the Registration,

Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation issued by the EU in 2007[42]. Hence, strawberry leaves may serve as a natural raw material and/or biocatalyst for the production of green leaf volatiles that are also reported to exhibit antimicrobial activity.

The analysis of cultivar-dependent phenolic content and profile is crucial for the quality control of strawberry-leaf based ingredients

The total phenolic contents of strawberry leaves were measured from three biological replicate samples per each cultivar (Fig.4.). The leaves of cultivar Salsa exhibited significantly higher total phenolic content than other cultivars, while Honeoye had significantly lower content. Inconsistent quality of natural products constitutes a fundamental problem in the supplement industry[1], and the results of the present study yet highlight the role of genetic control of leaf phenolic content and profile (Fig.3., Fig.4.).

Metabolomics approaches have been undertaken to analyze the drivers of the phytochemical composition and content in berry fruit plants. In a recent study employing a segregating raspberry population grown in two distinct environments identified that the polyphenols in general displayed evidence of tight genetic control but some specific compounds were environmentally influenced.[43] In grape vine (*Vitis vinifera*), a large variability between batches was observed when regarding the phenolic compositions of a leaf-based byproduct, and this variation was suggested to be due to the cultivar, growth cycle period, and processing conditions.[20] In this study, cultivars Florence and Salsa did not show any outstanding similarities when their whole metabolite profiles were considered, although they were grown in the same field and received identical horticultural management during the season (e.g. one-row system, no

irrigation, treatments with an insecticide and with a fungicide seven and two days before sample collection, respectively) (Fig.1).

The residues of agriculture and food industry have been the focus of intensive investigation over the last couple of decades, because there are ambitions 1) to increase the level of recycling to boost the sustainability of production chains; 2) to add value to agro-food- and non-food-byproducts; 3) to replace synthetic preservatives, antioxidants, and aromatics with more natural alternatives.[1,39] Today, consumers are aware of the functional possibilities of health-related products, and are in favor of natural ingredients in food products, nutraceuticals, pharmaceuticals, and cosmeceuticals.[1] Indeed, the protection of cells and tissues from stress (e.g. oxidative toxins etc.) is relevant not only in the food industry, but also in the pharmaceuticals and cosmetics sectors.[1] Food plant-derived extracts have shown potential as stable and non-irritating bioactive/functional ingredients in topical formulations when added in an appropriate concentration; furthermore, in non-food applications, the sometimes unpleasant taste of polyphenol-rich supplements does not constitute a problem.[1,11]

The economics of using horticultural coproducts need to be validated before practical uptake and exploitation can be undertaken. For example, drying, extraction, and quality control all come with associated costs largely dependent on the chosen method and the infrastructure at hand.[1] As strawberry leaves have a rather low water content (relative water content in leaves[44] and fruits[45] ca. 68% and 86%, respectively), they are light to transport and easy to dry. However this process needs to be controlled to limit, or ideally inhibit, dehydrative polymerization of polyphenols. Furthermore, the initially high total phenolic content should assure a reasonable phenolic concentration in strawberry leaf extracts, despite the possible losses during the extraction process.

In conclusion, the bioactive/functional compounds found in the leaves of different strawberry cultivars offer possible means for the selection of optimized leaf materials for food and for non-food applications. The results of this study indicate that with careful cultivar selection, the quantitative and qualitative metabolite profile of a strawberry leaf extract can be largely controlled and tailored to be used in specific added-value ingredients, supplements and non-food applications such as cosmetics stabilizers and antioxidants. Due to the consistent high levels of total phenolics and galloyl hexoses, cultivar Salsa would be an interesting target for antioxidant and antimicrobial studies, for instance. Cultivar Honeoye, although relatively low in total phenolics, may serve as a stable source of linolenic acid and possibly other octadecatrienoic acids for the development of natural odorants and preservatives. In addition, the bioactivities of strawberry-leaf derived caffeic acid derivatives would deserve further attention.

REFERENCES

1. Peschel W, Sánchez-Rabaneda F, Diekmann W, Plescher A, Gartzía I, Jiménez D, Lamuela-Raventos R, Buxaderas S and Codina C, An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chem* **97:** 137-150 (2006).
2. Teleszko M and Wojdyło A, Comparison of phenolic compounds and antioxidant potential between selected edible fruits and their leaves. *J Funct Foods* **14:** 736-746 (2015).
3. Yang W, Alanne A, Liu P, Kallio H and Yang B, Flavonol glycosides in currant leaves and variation with growth season, growth location and leaf position. *J Agric Food Chem* **63:** 9269-9276 (2015).
4. Hukkanen AT, Kokko HI, Buchala AJ, McDougall GJ, Stewart D, Kärenlampi SO and Karjalainen RO, Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries. *J Agric Food Chem* **55:** 1862-1870 (2007).

5. Kårlund A, Salminen J-P, Koskinen P, Ahern JR, Karonen M, Tiilikkala K and Karjalainen R, Polyphenols in strawberry (*Fragaria* × *ananassa*) leaves induced by plant activators. *J Agric Food Chem* **62:** 2592-2600 (2014).

6. Vagiri M, Conner S, Stewart D, Andersson SC, Verrall S, Johansson E and Rumpunen K, Phenolic compounds in blackcurrant (*Ribes nigrum* L.) leaves relative to leaf position and harvest date. *Food Chem* **172:** 135-142 (2015).

7. Oszmiański J, Wojdyło A, Gorzelany J and Kapusta, I, Identification and characterization of low molecular weight polyphenols in berry leaf extracts by HPLC-DAD and LC-ESI/MS. *J Agric Food Chem* **59:** 12830-12835 (2011).

8. Hokkanen J, Mattila S, Jaakola L, Pirttilä AM and Tolonen A, Identification of phenolic compounds from lingonberry (*Vaccinium vitis-idaea* L.), bilberry (*Vaccinium myrtillus* L.) and hybrid bilberry (*Vaccinium x intermedium* Ruthe L.) leaves. *J Agric Food Chem* **57:** 9437-9447 (2009).

9. Lavola A, Karjalainen R and Julkunen-Tiitto R, Bioactive polyphenols in leaves, stems, and berries of Saskatoon (*Amelanchier alnifolia* Nutt.) cultivars. *J Agric Food Chem* **60:** 1020-1027 (2012).

10. Buendía B, Gil MI, Tudela JA, Gady AL, Medina JJ, Soria C, López JM and Tomás-Barberán FA, HPLC-MS Analysis of proanthocyanidin oligomers and other phenolics in 15 strawberry cultivars. *J Agric Food Chem* **58:** 3916-3926 (2009).

11. Kårlund A, Hanhineva K, Lehtonen M, Karjalainen RO and Sandell, M, Nontargeted metabolite profiles and sensory properties of strawberry cultivars grown both organically and conventionally. *J Agric Food Chem* **63:** 1010-1019 (2015).

12. Giampieri F, Forbes-Hernandez TY, Gasparrini M, Alvarez-Suarez JM, Afrin S, Bompadre S, Quiles JL, Mezzetti B and Battino M, Strawberry as a health promoter: an evidence based review. *Food Funct* **6:** 1386-1398 (2015).

13. Tulipani S, Armeni T, Giampieri F, Alvarez-Suarez JM, Gonzalez-Paramás AM, Santos-Buelga C, Busco F, Principato G, Bompadre S and Quiles JL, Strawberry intake increases

blood fluid, erythrocyte and mononuclear cell defenses against oxidative challenge. *Food Chem* **156:** 87-93 (2014).

14. Bialasiewicz P, Prymont-Przyminska A, Zwolinska A, Sarniak A, Wlodarczyk A, Krol M, Glusac J, Nowak P, Markowski J and Rutkowski KP, Addition of strawberries to the usual diet decreases resting chemiluminescence of fasting blood in healthy subjects — possible health-promoting effect of these fruits consumption. *J Am Coll Nutr* **33:** 274-287 (2014).
15. Alvarez-Suarez JM, Giampieri F, Tulipani S, Casoli T, Di Stefano G, González-Paramás AM, Santos-Buelga C.; Busco F, Quiles JL and Cordero MD, One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular risk, oxidative stress markers and platelet activation in humans. *J Nutr Biochem* **25:** 289-294 (2014).
16. Wang SY and Lin H, Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J Agric Food Chem* **48:** 140-146 (2000).
17. Mudnic I, Modun D, Brizic I, Vukovic J, Generalic I, Katalinic V, Bilusic T, Ljubenkov I and Boban, M, Cardiovascular effects in vitro of aqueous extract of wild strawberry (*Fragaria vesca*, L.) leaves. *Phytomedicine* **16:** 462-469 (2009).
18. Ibrahim DS and Abd El-Maksoud MA, Effect of strawberry (*Fragaria× ananassa*) leaves extract on diabetic nephropathy in rats. *Int J Exp Pathol* **96:** 87-93 (2015).
19. Liberal J, Francisco V, Costa G, Figueirinha A, Amaral MT, Marques C, Girão H, Lopes MC, Cruz MT and Batista MT, Bioactivity of *Fragaria vesca* leaves through inflammation, proteasome and autophagy modulation. *J Ethnopharmacol* **158:** 113-122 (2014).
20. Monagas M, Hernández-Ledesma B, Gómez-Cordovés C and Bartolomé B, Commercial dietary ingredients from *Vitis vinifera* L. leaves and grape skins: antioxidant and chemical characterization. *J Agric Food Chem* **54:** 319-327 (2006).
21. Hanhineva K, Soininen P, Anttonen MJ, Kokko H, Rogachev I, Aharoni A, Laatikainen R and Kärenlampi S, NMR and UPLC-qTOF-MS/MS characterisation of novel phenylethanol derivatives of phenylpropanoid glucosides from the leaves of strawberry (*Fragaria* × *ananassa* cv. Jonsok). *Phytochem Anal* **20:** 353-364 (2009).

22. Everette JD, Bryant QM, Green AM, Abbey YA, Wangila GW and Walker RB, Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. *J Agric Food Chem* **58:** 8139-8144 (2010).

23. Hanhineva K, Rogachev I, Kokko H, Mintz-Oron S, Venger I, Kärenlampi S and Aharoni A, Non-targeted analysis of spatial metabolite composition in strawberry (*Fragaria* × *ananassa*) flowers. *Phytochemistry* **69:** 2463-2481 (2008).

24. Karaçelik AA, Küçük M, İskefiyeli Z, Aydemir S, De Smet S, Miserez B and Sandra P, Antioxidant components of *Viburnum opulus* L. determined by on-line HPLC–UV–ABTS radical scavenging and LC–UV–ESI-MS methods. *Food Chem* **175:** 106-114 (2015).

25. Farag MA, Mohsen M, Heinke R and Wessjohann LA, Metabolomic fingerprints of 21 date palm fruit varieties from Egypt using UPLC/PDA/ESI–qTOF-MS and GC–MS analyzed by chemometrics. *Food Res Int* **64:** 218-226 (2014).

26. Soukas A, Cohen P, Socci ND and Friedman JM, Leptin-specific patterns of gene expression in white adipose tissue. *Genes Develop* **14:** 963-980 (2000).

27. Fait A, Hanhineva K, Beleggia R, Dai N, Rogachev I, Nikiforova VJ, Fernie AR and Aharoni A, Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. *Plant Phys* **148:** 730-750 (2008).

28. Goossens Flevoplant. Rumba, a shiny start to the summer. Goossens Flevoplant B.V., PJ Ens, The Netherlands. URL: [http://www.flevoplant.nl/eng/vroeg_2_3427574760.pdf]; accessed May 2016.

29. Terao J, Murota K and Kawai Y, Conjugated quercetin glucuronides as bioactive metabolites and precursors of aglycone in vivo. *Food Funct* **2:** 11-17 (2011).

30. Day AJ, Bao Y, Morgan MR and Williamson G, Conjugation position of quercetin glucuronides and effect on biological activity. *Free Rad Biol Med* **29:** 1234-1243 (2000).

31. Calderón-Montaño JM, Burgos-Morón E, Pérez-Guerrero C and López-Lázaro M, A review on the dietary flavonoid kaempferol. *Mini-Rev Med Chem* **11:** 298-344 (2011).

32. Barve A, Chen C, Hebbar V, Desiderio J, Saw CL-L and Kong A-N, Metabolism, oral bioavailability and pharmacokinetics of chemopreventive kaempferol in rats. *Biopharm Drug Dispos* **30:** 356-365 (2009).

33. DuPont MS, Day AJ, Bennett RN, Mellon FA and Kroon PA, Absorption of kaempferol from endive, a source of kaempferol-3-glucuronide, in humans. *Eur J Clin Nutr* **58:** 947-954 (2004).

34. Aaby K, Mazur S, Nes A and Skrede G, Phenolic compounds in strawberry (*Fragaria x ananassa* Duch.) fruits: Composition in 27 cultivars and changes during ripening. *Food Chem* **132:** 86-97 (2012).

35. Espín JC, Larrosa M, Garcia-Conesa MT and Tomás-Barberán F, Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. *Evid Based Complement Alternat Med* **2013:** Article ID 270418 (2013).

36. Kuczkowia U, Petereit F and Nahrstedt A, Hydroxycinnamic acid derivatives obtained from a commercial *Crataegus* extract and from authentic *Crataegus* spp. *Sci Pharm* **82:** 835-846 (2014)

37. Mahesh V, Million-Rousseau R, Ullmann P, Chabrillange N, Bustamante J, Mondolot L, Morant M, Noirot M, Hamon S and de Kochko A, Functional characterization of two *p*-coumaroyl ester 3′-hydroxylase genes from coffee tree: evidence of a candidate for chlorogenic acid biosynthesis. *Plant Mol Biol* **64:** 145-159 (2007).

38. Lou Z, Wang H, Zhu S, Ma C and Wang Z, Antibacterial activity and mechanism of action of chlorogenic acid. *J Food Sci* **76:** M398-M403 (2011).

39. Gigot C, Ongena M, Fauconnier M, Wathelet J, Du Jardin P and Thonart P, The lipoxygenase metabolic pathway in plants: potential for industrial production of natural green leaf volatiles. *Biotechnol Agron Soc Environ* **14:** 451-460 (2010).

40. Nakamura S and Hatanaka A, Green-leaf-derived C6-aroma compounds with potent antibacterial action that act on both Gram-negative and Gram-positive bacteria. *J Agric Food Chem* **50:** 7639-7644 (2002).

41. Goers SK, Ghossi P, Patterson JT and Young CL, Process for producing a green leaf essence. *United States Patent* (1989).

42. Anonymous, Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency. *Off J EU* L 136/3 (2007).

43. Stewart D, McDougall GJ, Sungurtas J, Verrall S, Graham J and Martinussen I, Metabolomic approach to identifying bioactive compounds in berries: advances toward fruit nutritional enhancement. *Mol Nutr Food Res* **51:** 645-651 (2007).

44. Keutgen AJ and Pawelzik E, Apoplastic antioxidative system responses to ozone stress in strawberry leaves. *J Plant Phys***165:** 868-875 (2008).

45. Gulen H and Eris A, Some physiological changes in strawberry (*Fragaria* × *ananassa* `Camarosa') plants under heat stress. *J Hort Sci Biotechnol* **78:** 894-898 (2003).

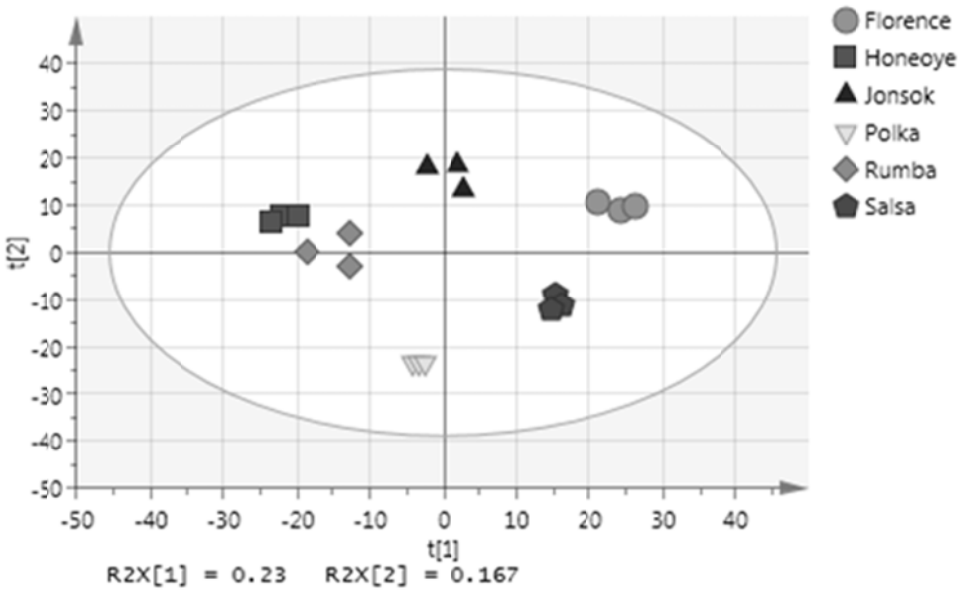

Figure 1. Principal component analysis (PCA) of the metabolite contents in the leaves of six strawberry cultivars. The PCA plot shows differences between leaf sample replicates according to their metabolite profiles based on metabolite-specific signal abundances. The analysis is conducted on the basis of 1168 molecular features that were present in all three sample replicates of at least one cultivar. Each replicate is a pool of 10 strawberry leaves. t[1], PC1; t[2], PC2; R2X, explained variation.

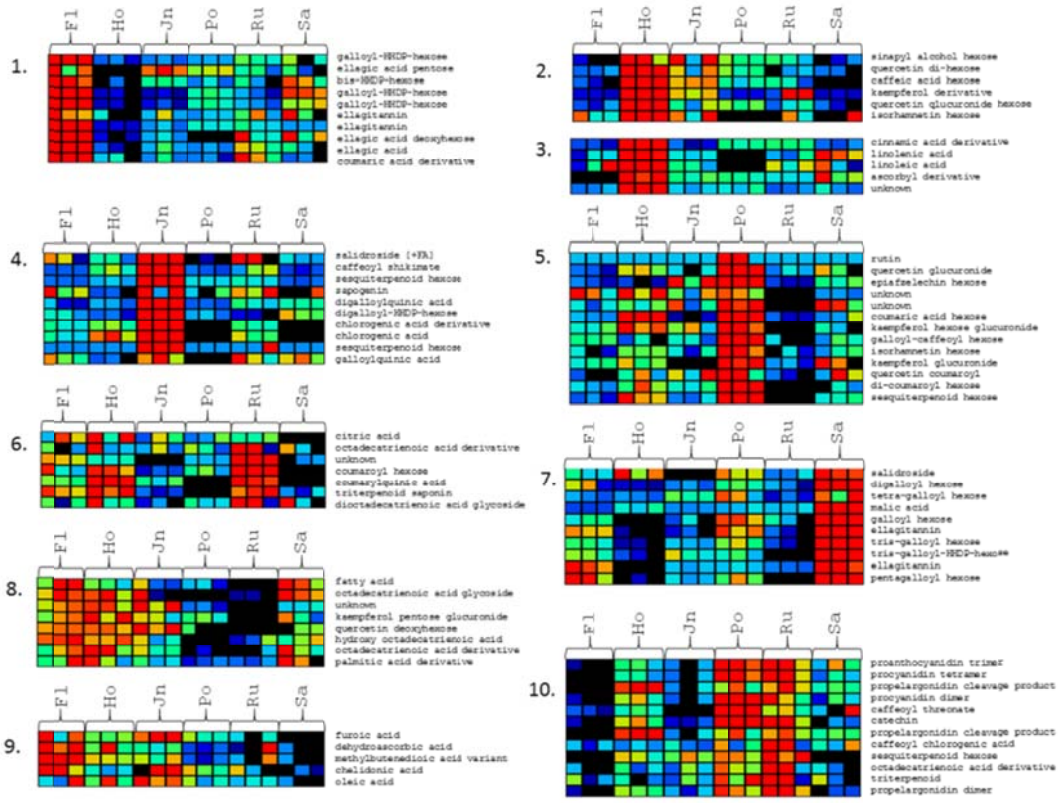

Figure 2. Heat map representation of the k-means clustering analysis based on the normalized signal abundances of the 84 tentatively identified strawberry leaf metabolites across all the analyzed samples representing six different cultivars (Fl, Florence; Ho, Honeoye; Jn, Jonsok; Po, Polka; Ru, Rumba; Sa, Salsa). Metabolites having similar accumulation patterns are classified in clusters (1-10). The color-coding scale indicates the relative abundance within each metabolite: blue/black: low abundance, red: high abundance, green/yellow: average abundance. A sample replicate is a pool of 10 strawberry leaves. FA, formic acid adduct.

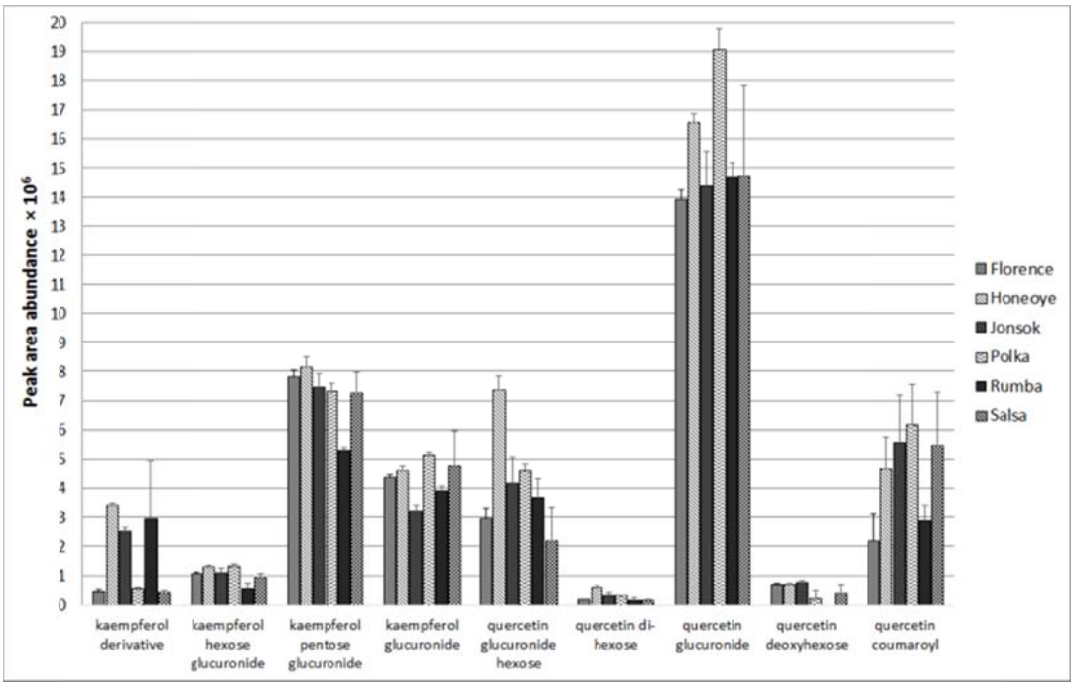

Figure 3. The mean peak area abundance values (+SD) of different quercetin and kaempferol derivatives in the leaves of six strawberry cultivars.

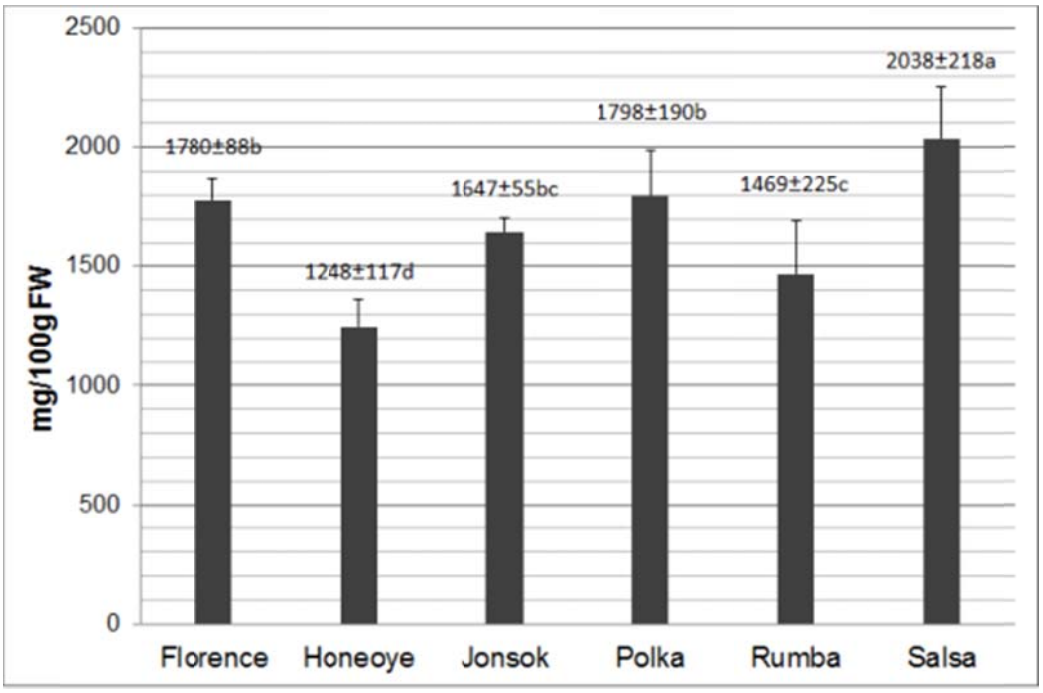

Figure 4. The mean total phenolic content (+SD) in the leaves of six strawberry cultivars. Contents with the same letter (a-d) do not differ significantly between cultivars.

Table 1. Metabolites Tentatively Identified from Strawberry Leaves by LC-qTOF-MS and MS/MS Analysis. Data Were Acquired in Negative Electrospray Ionization (ESI–). t_R, retention time; MW, molecular weight; NA, not available; FA, formic acid adduct.

No.	t_R (min)	MW	[M-H]	MS/MS	ID	Reference
1	1.0861	174.01653	173.0101	129.0185, 111.0066, 85.0296	dehydroascorbic acid	11, METLIN
2	1.1207	130.02638	129.0199	116.9357, 85.0290	methylbutenedioic acid variant	11
3	1.1222	192.02711	191.0186	173.0080, 111.0086	citric acid	11
4	1.3238	134.05775	133.0129	115.0032, 71.0137	malic acid	11
5	1.5706	344.0753	343.0675	191.0560, 169.0137, 93.0331	galloylquinic acid	METLIN
6	1.6362	184.0005	182.9936	139.0031, 111.0080, 95.0141, 83.0129	chelidonic acid	SciFinder
7	1.6646	332.0752	331.1012	169.0503, 123.0445	galloyl hexose	23
8	1.6788	112.0161	111.0071	67.0190	furoic acid	11
9	1.9746	784.0763	783.0707	481.0581, 300.9949	bis-HHDP-hexose	23
10	2.0975	634.0815	633.0764	481.0556, 463.0571, 301.0002	galloyl-HHDP-hexose	23
11	2.1179	302.06467	301.0574	283.0449, 168.0062, 149.9952, 125.0243	unknown*)	11
12	2.2183	634.0810	633.0741	300.9975	galloyl-HHDP-hexose	23
13	2.3067	316.08017	315.0740	153.0160, 123.0466, 109.0293	unknown*)	11
14	2.4000	578.1445	577.1362	425.0909, 407.0766, 289.0715	procyanidin dimer	23

15	2.4028	866.20764	865.1974	575.1197, 287.0560	proanthocyanidin trimer	11
16	2.5251	634.0814	633.0645	463.0500, 300.9991	galloyl-HHDP-hexose	23
17	2.5582	496.0863	495.0790	NA	digalloylquinic acid*)	23
18	2.5914, 2.7056	300.08524, 346.12735	299.1139, 345.1220 [M-H+FA]	179.0564, 137.0618, 89.0232, 59.0137	salidroside	11
19	2.6472	1154.2687	1153.2620	NA	procyanidin tetramer*)	23
20	2.6543	342.1000	341.0849	179.0320, 161.0221	caffeic acid hexose	23
21	2.6922	298.0697	297.0625	135.0297, 89.0227, 75.0088	caffeoyl threonate	MET LIN
22	2.7195	290.0797	289.0727	245.0826, 221.0831, 203.0713, 109.0294	catechin	23
23	2.7243	176.06854	175.0249	115.0033, 87.0088, 71.0139, *463.0905*	ascorbyl derivative	11, MET LIN
24	2.7620	562.1482	561.1399	NA	propelargonidin dimer*)	23
25	2.7749	372.1068	371.1333	209.0826, 191.0679, 149.0610	sinapyl alcohol hexose	MET LIN
26	2.7800	484.0866	483.0806	331.0689, 271.0471, 169.0132	digalloyl hexose	23
27	2.9459	786.0925	785.0881	615.0615, 300.9983	digalloyl-HHDP-hexose	23
28	3.0186	708.1925	707.1825	353.0889, 191.0566	chlorogenic acid derivative	24
29	3.0582	418.11182	417.1046	285.0600, 241.0698, 163.0388, 152.0110	unknown*)	11
30	3.1549	326.10114	325.0933	307.0819, 163.0399, 145.0296	coumaric acid hexose	11
31	3.1977	354.0955	353.0892	191.0566, 173.0458, 135.0453	chlorogenic acid	23
32	3.2246	756.1753	755.1687	NA	unknown*)	23
33	3.2444	936.0881	935.0801	NA	ellagitannin*)	23
34	3.2612	636.0972	635.0932	465.0689, 313.0546, 169.0131	tris-galloyl hexose	23
35	3.3000	292.0224	291.0157	247.0288, 163.0413, 147.0457, 117.0344	coumaric acid derivative	11
36	3.3108	640.1303	639.1214	463.0895, 301.0365	quercetin glucuronide hexose	23
37	3.4672	1236.07955	1235.0671	933.0596. 299.9906	ellagitannin	23
38	3.4734	938.1057	937.0951	767.0719, 300.9986	tris-galloyl-HHDP-hexose	23

39	3.5110	466.11182	465.1032	447.0832, 285.0388, 241.0457, 151.0024	kaempferol derivative	11
40	3.5893	626.1500	625.1420	463.0884, 301.0362	quercetin di-hexose	23
41	3.7545	788.1079	787.1055	617.0795, 465.0676, 271.0321, 169.0150	tetragalloyl hexose	23
42	3.7634	336.0859	335.0775	161.0243, 111.0449, 93.0349	caffeoyl shikimate	MET LIN
43	3.8186	356.1118	355.1016 [M-H+FA]	309.0976 [M-H], 147.0450, 103.0559	cinnamic acid hexose	11
44	3.8395, 3.8398	332.09055, 376.0803	331.0815, 375.0740	289.0733, 271.0612, 125.0243, *751.1517, 245.0824*	propelargonidin	11
45	3.8424	308.0931	307.0883	145.0272	coumaroyl hexose	23
46	3.8779	1086.0837	1085.0723	NA	ellagitannin*)	23
47	3.9134	338.1013	337.0805	191.0540, 173.0438, 85.0293	coumaroylquinic acid	MET LIN
48	4.0013	624.1348	623.1298	337.0794, 285.0420, 113.0258	kaempferol hexose glucuronide	23
49	4.0392	1086.0845	1085.0761	NA	ellagitannin*)	23
50	4.1796	940.1205	939.1094	769.0850, 617.0669, 169.0087	pentagalloyl hexose	23
51	4.2438	610.1183	609.1121	301.0352, 227.0342	rutin	23
52	4.4771	494.1075	493.0634	NA	galloyl-caffeoyl hexose*)	23
53	4.5372	448.1021	447.1292	NA	unknown*)	11
54	4.6879	434.04922	433.0379	299.9898, 283.9898	ellagic acid pentose	23
55	4.7052	594.1244	593.1163	307.0661, 285.0413, 113.0242	kaempferol pentose glucuronide	23
56	4.7121	516.1287	515.1192	353.0871, 191.0554, 135.1960	dicaffeoyl quinic acid	23, MET LIN
57	4.7683	478.0763	477.0709	301.0361, 255.0300, 178.9983, 151.0035, 121.0300	quercetin glucuronide	23
58	4.8550	448.06552	447.0584	300.9994, 257.0115, 229.0145, 200.0048	ellagic acid deoxyhexose	11
59	4.9626	302.0067	300.9954	283.9964, 200.0113, 145.0288, 101.0410	ellagic acid	23, 11
60	5.2400	478.1074	477.1006	314.0455, 301.0366, 255.0322	isorhamnetin hexose	23
61	5.2730	462.0813	461.0692	285.0398, 175.0237, 113.0233	kaempferol glucuronide	11
62	5.2962	896.2022	895.1967	447.0920, 301.0344, 272.0338, 178.9943	quercetin deoxyhexose	23, 11

63	5.3564	478.1073	477.1037	314.0424, 301.0346, 243.0268, 178.9987	isorhamnetin hexose	23
64	5.4848	436.10193	435.1285	273.0761, 167.0343	epiafzelechin hexose	11
65	5.8327	446.1593	445.1488	301.0363, 163.0380, 145.0286	quercetin coumaroyl	23
66	6.0302	472.1383	471.1307	163.0397, 145.0284	di-coumaroyl hexose	23
67	6.5148	464.2637	463.2568 [M-H+FA]	417.2509 [M-H], 255.1928, 161.0444	sesquiterpenoid hexose	23, 11
68	6.7400	464.26276	463.2550 [M-H+FA]	417.2463 [M-H], 255.1844, 161.0466	sesquiterpenoid hexose	23, 11
69	6.7921	464.2628	463.2540 [M-H+FA]	417.2514 [M-H], 255.1645, 161.0445, 101.0230	sesquiterpenoid hexose	23, 11
70	6.9027	464.2630	463.2569 [M-H+FA]	417.2493 [M-H]	sesquiterpenoid hexose	23, 11
71	7.2904	328.2253	327.2175	309.2099, 283.0627, 255.3553, 171.1034	fatty acid	25, MET LIN
72	7.8384	504.3454	503.3391	485.3279	triterpenoid saponin	23
73	8.8041	488.35153	487.3432	469.3330, 407.3308, 135.0753	sapogenin	11
74	9.1273	294.2201	293.2123	275.2018, 171.1025	hydroxyoctadeca -trienoic acid	25
75	9.3914	722.3755	721.3656	675.3608, 397.1346, 277.2174, 235.0795	octadecatrienoic acid derivative	MET LIN
76	9.6247	560.3220	559.3144	513.3085, 277.2188, 253.0934, 161.0418	octadecatrienoic acid glycoside	MET LIN
77	10.0863	541.33966	540.3286	480.3092, 255.2323	palmitic acid derivative	11
78	10.2906	278.2251	277.2179	233.2248, 59.0139	linolenic acid	MET LIN
79	10.5074	280.24063	279.2355	71.0117	linoleic acid	11, MET LIN
80	10.7280	282.2561	281.2489	126.573	oleic acid	11
81	11.1404	982.5884	981.5764	935.5769, 657.3502, 397.1357, 277.2176	dioctadecatrienoic acid glycoside	MET LIN
82	11.3536	820.5347	819.5286	513.3087, 277.2178	octadecatrienoic acid derivative	MET LIN
83	11.4462	820.5369	819.4226	513.3086, 277.2176	octadecatrienoic acid derivative	MET LIN
84	11.5011	622.4460	621.4382	486.8462, 271.7586, 153.4179	triterpenoid	11

*) Qualified or/and identified on the basis of previously reported molecular weight and retention time.